STIC-ILL

NO

467391

From: STIC-Biotech/ChemLib
Sent: Thursday, October 09, 2003 6:01 AM
To: STIC-ILL
Subject: FW: In re: 10/005, 510 Journal article

-----Original Message-----
From: Ford, Vanessa
Sent: Wednesday, October 08, 2003 5:49 PM
To: STIC-Biotech/ChemLib
Subject: In re: 10/005, 510 Journal article

11919556

Please supply:

SO VET.MED. (76, NO.8, 1185-86, 1981).*

SO Vet.Med. (82, No. 6, 646-50, 1987) 2 Tab. 23 Ref. *

SO VETERINARY PARASITOLOGY, (MAY 1993) Vol. 47, No. 3-4, pp. 225-233.

SO Z PARASITENKD, (1983) 69 (1), 27-34.*

SO Revista Brasileira de Parasitologia Veterinaria, (1995) Vol. 4, No. 1, pp.* 15-19.

SO Vet.Rec. (111, No. 18, 414-15, 1982) 2 Fig. 2 Tab. 12 Ref.*

SO J PROTOZOOL, (1987) 34 (4), 398-402.

SO AM J TROP MED HYG, (1987) 36 (3), 505-508*

Applied and Environmental Microbiology, (1995) Vol. 61, No. 2, pp.*

SO Journal of Parasitology, (Feb., 1998) Vol. 84, No. 1, pp. 8-15.

SO Journal of the Egyptian Society of Parasitology, (Aug., 1999) Vol. 29, No. 2, pp. 551-560. *

SO FEMS Microbiology Letters, (1994) Vol. 118, No. 1-2, pp. 45-49.

SO J.Protozool. (35, No. 4, 583-89, 1988) 7 Fig. 2 Tab. 20 Ref. *

SO AM J TROP MED HYG, (1987) 36 (3), 505-508. *

SO J PARASITOL, (1976) 62 (2), 199-202.

Vanessa L. Ford
Biotechnology Patent Examiner
Office: CM1 8A16
Mailbox: CM1 8E12
Phone: 703.308.4735
Art Unit: 1645

J. Protozool., 34(4), 1987, pp. 398–402

Studies of In Vitro Excystation of *Cryptosporidium parvum* from Calves[1]

DOUGLAS B. WOODMANSEE[2]

Departments of Zoology and Veterinary Pathology, Iowa State University, and U.S. Department of Agriculture, Agricultural Research Service, National Animal Disease Center, Ames, Iowa 50010

ABSTRACT. Studies of in vitro excystation of *Cryptosporidium parvum* from calves showed that sporozoite yields were optimum when oocysts were treated with sodium hypochlorite, then incubated at 37°C for 60 min in the presence of taurocholic acid solutions at pH about 7.0. Trypsin was not required for excystation and high concentrations were inhibitory. Studies using protease inhibitors and direct assays for proteolysis failed to implicate proteolytic enzymes as effectors of excystation. The results suggest that *Cryptosporidium* uses excystation mechanisms that are different from those used by *Eimeria* spp.

CRYPTOSPORIDIUM parvum (Apicomplexa, Coccidiasina) is a parasite of mucosal surfaces of many host species (17). It is believed to be a significant cause of diarrhea in humans (3) and domestic animals (7). The life cycle is direct and infection is transmitted by the ingestion of oocysts that subsequently release sporozoites by a process of excystation (2).

In vitro excystation by coccidia of the genus *Eimeria* has been well studied and shown to be a biphasic process. In the first phase, the oocyst wall is either mechanically disrupted or opened by the action of reducing agents and CO_2. In the second phase, sporozoites are released from sporocysts by treatment with digestive enzymes and surface-active agents such as detergents or bile salts (13, 19). The *Eimeria* oocyst and sporocyst walls are interrupted by the presence of a micropyle and Stieda body, which are removed during phases 1 and 2, respectively. Other coccidians differ morphologically from *Eimeria*, releasing sporozoites through sutures in their cyst walls (1). In the case of *Cryptosporidium*, sporocysts are absent and the suture is in the oocyst wall (2). In *Sarcocystis* and *Toxoplasma*, the oocyst wall lacks morphological specializations and the suture is in the sporocyst wall (1). It has been thought that excystation mechanisms for suture-bearing coccidia are similar to those of *Eimeria* because in vitro excystation procedures that work for *Eimeria* also work for suture bearers.

In the present study, experiments were done to determine optimum conditions for in vitro excystation of *Cryptosporidium* and to examine the mechanisms of excystation. The results suggest that excystation mechanisms for *Cryptosporidium*, and perhaps other suture-bearing coccidians, differ from those of *Eimeria* spp.

MATERIALS AND METHODS

Oocysts. Feces containing *Cryptosporidium parvum* oocysts were collected from experimentally infected calves, sieved, mixed with $K_2Cr_2O_7$, and stored as previously described (6). Oocysts were isolated by layering 25 ml of feces over sucrose step gradients made up of 25 ml of Sheather's sucrose solution (14) diluted to a specific gravity of 1.18 g/ml, covered by 50 ml of Sheather's diluted to 1.09 g/ml, covered by 25 ml of Sheather's at 1.02 g/ml. Gradients were centrifuged for 15 min at 900 *g* in a swinging bucket rotor. The 1.09 g/ml fraction was collected and diluted in Dulbecco's phosphate-buffered saline solution (PBS). The oocysts were pelleted by centrifugation at 900 *g* for 10 min and stored in 2.5% $K_2Cr_2O_7$ at 4°C until used (up to two months).

Data collection and handling. After excystation, oocysts, sporozoites, and cast oocyst walls were counted in a phase contrast hemacytometer using Nomarski interference contrast microscopy. One-square-millimeter areas were counted until the number of oocysts and cast walls totaled at least 100. Percent excystation was calculated by dividing the number of empty cyst walls by the sum of the cyst walls and unexcysted oocysts. Sporozoite/cyst wall ratio was calculated by dividing the number of sporozoites by the number of cyst walls. The theoretical value of this ratio is four because each oocyst contains four sporozoites (16). Percent theoretical sporozoite yield was calculated by dividing the number of sporozoites observed by 4× the sum of the number of cyst walls and the number of unexcysted oocysts. Experiments were performed in triplicate unless otherwise noted. Standard errors for points on graphs ranged from ±2 to ±15, but most points had errors within 15% of the value of the mean.

Excystation assays. Experiments to determine optimum excystation procedures used oocysts that were washed 3× in cold (0°C) PBS and once in 3 ml of cold test fluid. Pellets were resuspended in 1 ml of test fluid at room temperature and placed in a 37°C water bath. Tubes were removed to an ice bath after the appropriate incubation time. Bleach treatment, when used, consisted of resuspending washed oocysts in full-strength commercial bleach (5.25% sodium hypochlorite) in an ice bath for 5 min, then washing 3× in cold PBS. The taurocholic acid (CTA) used was crude taurocholic acid, sodium salt (Sigma Chem. Co., St. Louis, MO). Trypsin was the "1:250" trypsin of Difco (Difco Labs., Detroit, MI).

To study further the effects of trypsin on excystation, oocysts were treated with bleach, resuspended in test solutions in centrifuge tubes, and placed in a 37°C water bath. Tubes were removed to an ice bath at 5-min intervals from 0 to 30 min. All test solutions contained 0.15% CTA. To test reversibility of trypsin inhibition, bleach-treated oocysts were incubated with 5% trypsin in PBS for 20 min at 37°C, washed 3× in PBS, then assayed for excystation in 0.15% CTA.

To compare the effects of bleach and cysteine/CO_2 pretreatments, one group of washed oocysts was suspended in 0.02 M cysteine HCl (Sigma) in 0.85% saline. The suspension was overlaid with a 50% mixture of CO_2 and air, then sealed and incubated at 37°C for 16 h. A second group of washed oocysts was mixed with saline, overlaid with air, and incubated. After incubation, both groups were divided into two parts; one treated with bleach and washed in PBS, and the other washed in PBS. All groups were suspended in 0.15% CTA in test tubes and excysted at 37°C. Tubes were removed to an ice bath at intervals and scored.

To test the effects of enzyme inhibitors on excystation, bleach-treated oocysts were resuspended to a concentration of 1.1×10^7 oocysts/ml and held on ice. Fifty microliters of inhibitor solution or PBS and 50 µl of 0.33% CTA were placed into test tubes in a 37°C water bath and allowed to warm for about 5 min. Ten microliters of oocyst suspension were added to each

[1] This study was supported by the U.S. Department of Agriculture, cooperative agreement #58-519B-2-1160 and by a fellowship provided by the J. E. Salsbury Foundation. The author wishes to thank Ms. Deborah Hammen for technical assistance. No endorsements are herein implied.

[2] Address correspondence to D. B. Woodmansee, U.S. Department of Agriculture, Agricultural Research Service, National Animal Disease Center, P.O. Box 70, Ames, Iowa 50010.

TABLE I. *Excystation of* Cryptosporidium *oocysts exposed to different excystation solutions.*

Basc solution	[CTA][a]	[TRYP][b]	Time[c]	Mean excys-tation	Mean % sporozoite cyst wall ratio	n
PBS[d]	1.5	0.5	30	56	1.54	4
PBS	1.5	0	30	52	2.62	4
PBS	0.15	0	30	38	4.11	3
PBS	0	0	30	0	—	3
H_2O	0	0	60	2	0.33	3

[a] Concentration (% w/v) of crude taurocholic acid.
[b] Concentration (% w/v) of trypsin.
[c] In minutes.
[d] Dulbecco's phosphate-buffered saline.

tube at 5-min intervals for 30 min. Immediately after addition of oocysts to the last tube, all tubes were removed to an ice bath and % excystation determined. Inhibitors used (all purchased from Sigma) were phenylmethylsulfonyl fluoride (PMSF), a noncompetitive inhibitor of serine proteases; N-α-p-tosyl-L-arginine methyl ester (TAME), a competitive substrate analog for serine proteases; N-α-p-tosyl-L-lysine chloromethyl ketone (TLCK), a noncompetitive inhibitor of serine proteases with specificity for trypsin; N-tosyl-L-phenylalanine chloromethyl ketone (TPCK), a compound similar to TLCK but with specificity for chymotrypsin; pepstatin, a pepsin inhibitor; and leupeptin, a competitive inhibitor of both trypsin and papain. The PMSF and TPCK were made up as stock solutions in ethanol and methanol, respectively, and diluted with PBS prior to use.

Azocoll assays. Bleach-treated oocysts (1×10^7 oocysts/ml) were suspended in 0.15% CTA in 0.1 M phosphate buffer (PB), pH 7.0, and excysted for 45 min at 37°C. Azocoll (Calbiochem, San Diego, CA), a general proteolytic substrate, was added to a concentration of 5 mg/ml to PB, 0.1 M acetate buffer, pH 5.0, or 0.1 M Tris, pH 9.0, in 1.5-ml microfuge tubes. One hundred microliters of excysted oocysts or PB was added to the microfuge tubes and they were incubated at 37°C for 24 h. After incubation, tubes were centrifuged at 6630 *g* for 2 min. Supernatants were collected, and absorbance at 520 nm was determined with a spectrophotometer.

RESULTS

Table I shows results of preliminary experiments to examine the effects of trypsin and CTA on excystation. Trypsin was found to be unnecessary for excystation and may have been detrimental to sporozoite survival. Reduction of CTA concentration decreased excystation but increased sporozoite survival. Little or no excystation was observed in the absence of CTA.

Experiments to determine optimal sporozoite excystation conditions were designed on the basis of the preliminary experiments. Figure 1 shows the results of time course experiments using oocysts exposed to 0.15% CTA. Excystation occurred rapidly between 15 and 60 min but leveled off thereafter. Sporozoite/cyst wall ratios were impossible to interpret at 0 and 15 min because of the small numbers of sporozoites observed, but were close to the theoretical value of four at 30 min. The steady decline in the ratio after 30 min was due to disappearance of sporozoites. If samples were placed in an ice bath after 30 min, excystation stopped and sporozoite/cyst wall ratios stabilized. Calculation of sporozoite yields from the data from Fig. 1 shows that one hour of excystation gave the best sporozoite yield (Fig. 2).

The effect of pH on excystation was examined by exposing oocysts at 37°C for one hour to 0.15% CTA solutions adjusted with HCl or NaOH (Fig. 3). Higher excystation rates were ob-

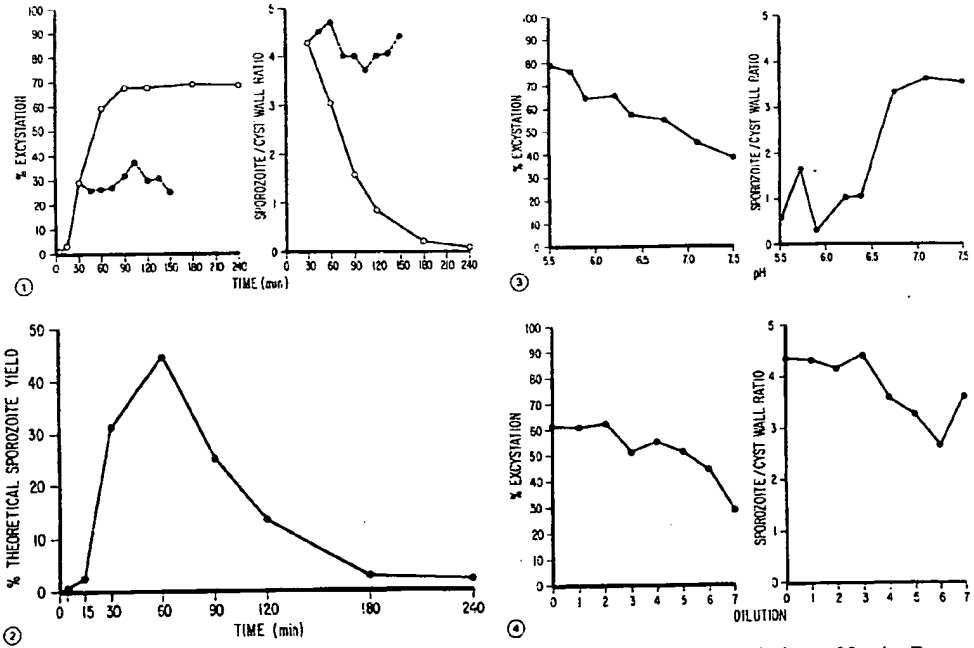


Fig. 1–4. 1. Time course for excystation of oocysts. Dashed lines represent values for tubes placed in ice at 30 min. Excystation conditions: 0.15% CTA in PBS, 37°C. 2. Sporozoite yield calculated from data in Fig. 1. 3. Effects of pH on excystation of oocysts. Excystation conditions: 0.15% CTA, 37°C, 60 min. 4. Effects of CTA concentration on excystation of oocysts. Dilutions are two-fold dilutions of 1.5% CTA in PBS. Excystation conditions: 37°C, 60 min, pH 7.0.

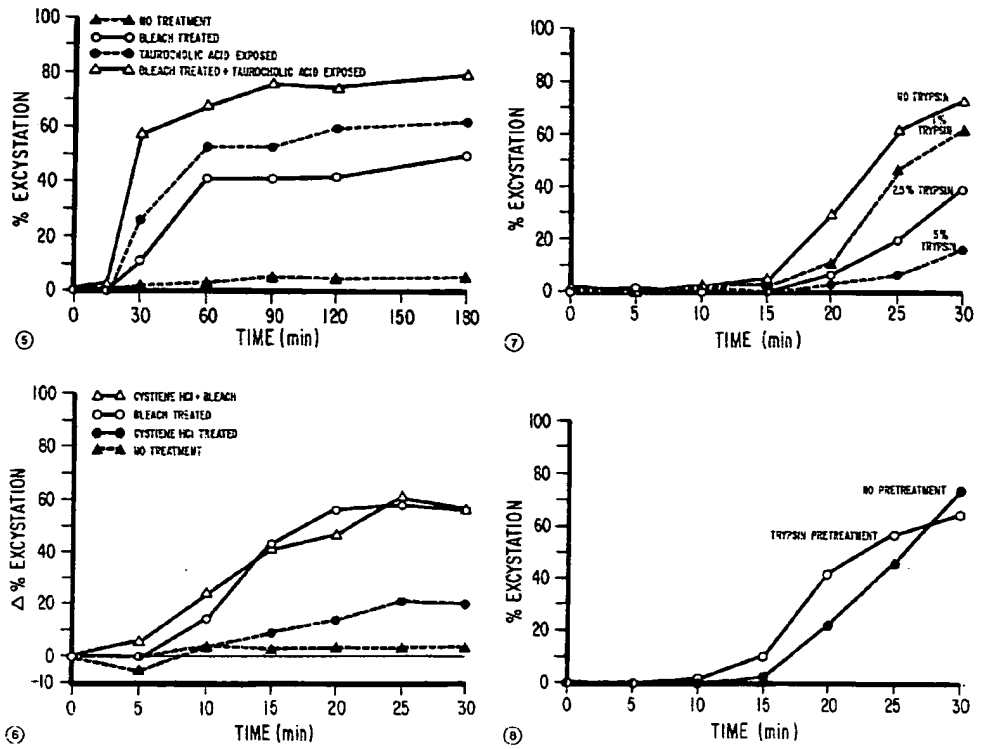


Fig. 5–8. 5. Effects of bleach treatment on excystation in the presence and absence of 0.15% CTA. Excystation conditions: 37°C, pH 7.0. 6. Effect of cysteine HCl and CO_2 pretreatment compared to bleach treatment. Excystation conditions: 0.15% CTA, 37°C, pH 7.0. 7. Effect of trypsin on excystation of bleach-treated oocysts. Excystation conditions as in Fig. 6. 8. Reversibility of trypsin effects. Oocysts were pretreated by incubation with 5% trypsin for 20 min at 37°C, then washed 3× in PBS. Excystation conditions as in Fig. 6.

served at lower pH, but sporozoite survival was poor at pH ≤6.5.

Oocysts exposed to two-fold serial dilutions of 1.5% CTA were incubated at 37°C for one hour to determine the effects of taurocholic acid concentration (Fig. 4). Substantial excystation occurred over a wide range of concentrations, but values tended to decline as the concentration was reduced. Sporozoite/cyst wall ratios similarly declined as CTA concentrations were reduced.

The following experiments were performed to examine effects of various pretreatments on excystation rather than to optimize sporozoite yields; thus, data on sporozoite survival were not collected. Figure 5 shows the results of comparisons of bleach-treated and non-bleach-treated oocysts in the presence and absence of taurocholic acid. Bleach treatment enabled excystation to occur in the absence of CTA. Highest levels of excystation were observed when bleach treatment was combined with taurocholic acid exposure.

Figure 6 shows the effect on excystation of exposure of oocysts to cysteine HCl and CO_2. Data are expressed as change in % excystation (excystation value at time 0 subtracted from excystation values for each subsequent time), because spontaneous excystation of 5–10% was observed after incubation for 16 h at 37°C either in the presence or absence of cysteine/CO_2. Treatment of oocysts with cysteine/CO_2 increased excystation rates upon exposure to 0.15% CTA compared to controls that were incubated in saline. The combination of cysteine/CO_2 and bleach treatments did not increase excystation rates over bleach treatment alone.

Figures 7 and 8 show results of experiments to examine further the potential role of trypsin in excystation. Addition of 10–50 mg/ml of trypsin to 0.15% CTA reduced excystation in a dose-dependent manner (Fig. 7). Inhibition by trypsin was found to be reversible (Fig. 8) and could not be mimicked by equivalent concentrations of bovine serum albumin (data not shown).

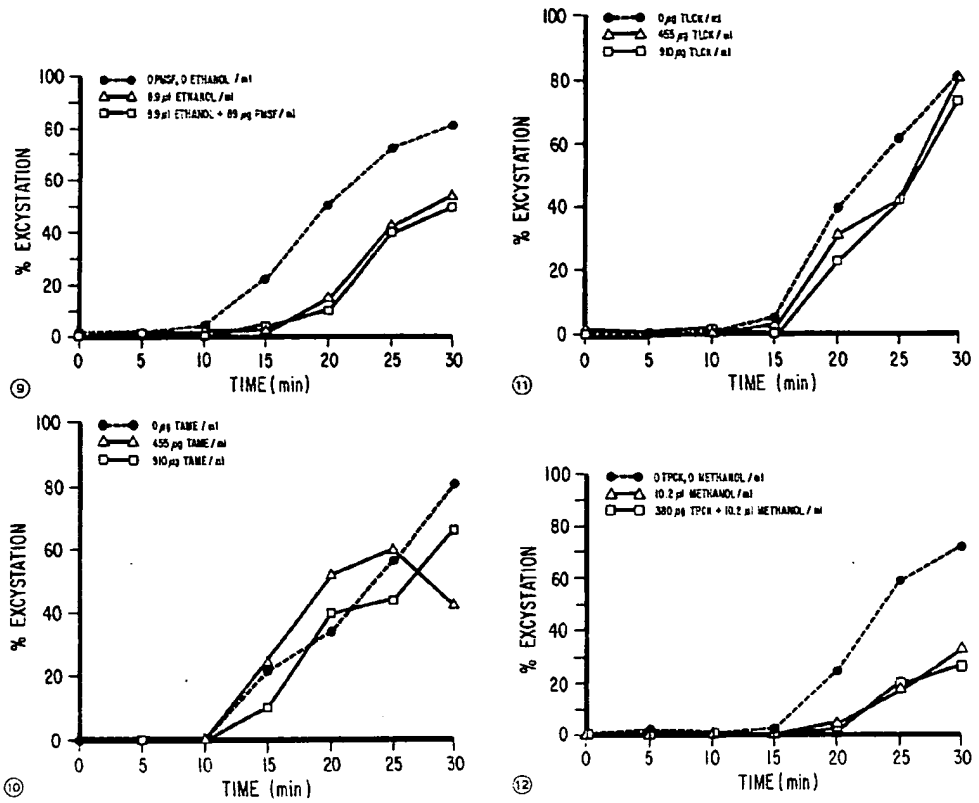
The PMSF, TAME, TPCK, TLCK, leupeptin, and pepstatin did not appear to inhibit excystation (Figs. 9–14), but ethanol and methanol were inhibitory (Figs. 9, 12). Experiments using Azocoll failed to detect proteolytic activity in CTA solutions or in CTA solutions containing excysted oocysts (Table II).

DISCUSSION

From these results, it is possible to suggest efficient conditions for in vitro excystation of *Cryptosporidium parvum*. Taurocholic acid is required unless the oocysts are bleach-treated, but concentrations can be varied over a wide range with little effect on sporozoite recovery. Best excystation is achieved when bleach pretreatment is combined with taurocholic acid exposure. Sixty-minute incubations at 37°C, pH 7.0, gave the best results in this study.

Sporozoites were short-lived at 37°C, and placement of the sporozoites in an ice bath after excystation stabilized sporozoite numbers. This result demonstrates that excystation and sporozoite loss are temperature dependent and that holding the samples in an ice bath until they were scored did not affect the outcome of the kinetic experiments. In a previous investigation, sporozoite loss to a far lesser extent was observed after excystation (11).

Examination of the kinetics of excystation consistently revealed a lag time of 10–15 min before excystation began. Ex-



Figs. 9–12. Effects of protease inhibitors on excystation of bleach-treated oocysts. Excystation conditions as in Fig. 6. 9. PMSF in ethanol and ethanol alone. 10. TAME, experiment similar to Fig. 9. 11. TLCK, experiment similar to Fig. 9. 12. TPCK in methanol and methanol alone, experiment similar to Fig. 9.

cystation occurred rapidly from 15 to 60 min and leveled off by 90 min. In the only experiment in which lag time was reduced (Fig. 6), the oocysts were incubated at 37°C overnight prior to the experiment, which seemed to change the oocyst's reactivity to taurocholic acid. The consistent appearance of the lag under standard conditions suggests that it is a consequence of the excystation mechanism.

These experiments reinforce the role of bile salts as a trigger for excystation of *Cryptosporidium*. The conclusion of Fayer & Leek (5) that excystation can occur without host stimuli was not supported, except in the case of bleach-treated oocysts. These results replicate and extend those of Reduker & Speer (11) and Reduker et al. (12), who concluded that the spontaneous excystation observed by Fayer & Leek (5) was probably due to oocyst degradation. The observation of multiplicative effects between bleach and taurocholic acid suggest that the two stimuli work via different mechanisms, a conclusion consistent with ultrastructural data that show that the outer layers of the oocyst wall are removed by bleach but not by taurocholic acid (12).

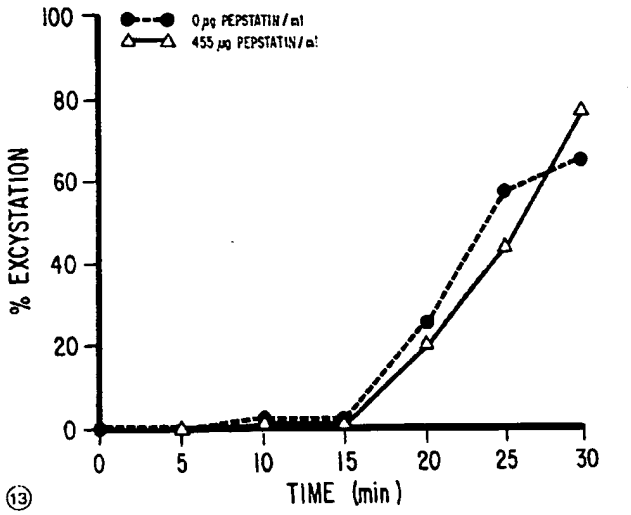
Failure of bleach and cysteine/CO_2 pretreatments to show additive effects suggests that they operate via the same mechanism. Cysteine/CO_2 increases the number of sulfhydryl groups on the exterior of *Eimeria* oocysts (10) and is thought to mimic stomach passage in vivo (9). Bleach treatment may also mimic some process that occurs in the stomach.

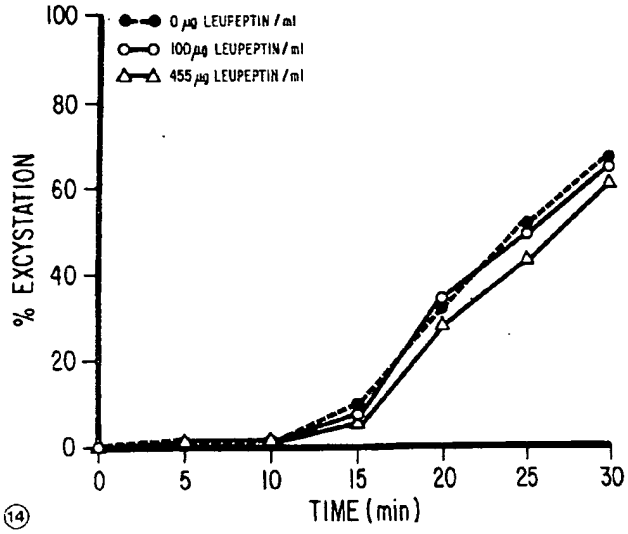
The observations that trypsin is unnecessary for excystation and that high concentrations are inhibitory were somewhat unexpected. Earlier papers describing excystation of *Cryptosporidium* consistently included trypsin as part of the excystation fluid (5, 11, 12, 18, 20), but more recent reports (11, 15) have described excystation in the absence of exogenous proteases. The observation that solutions of taurocholic acid, with or without excysted oocysts, failed to cleave Azocoll argues against the hypothesis that proteases in excystation fluids are responsible for excystation in vitro. Since exogenous proteolytic enzymes are believed essential to the excystation of *Eimeria* (13), the excystation mechanism used by *Cryptosporidium* would appear to be fundamentally different. Fayer & Leek (4) observed excystation of *Sarcocystis fusiformis* sporocysts in the absence of

TABLE II. *Results of incubation of Azocoll with 0.15% CTA or 0.15% CTA containing 1 × 10⁶ excysted* Cryptosporidium *oocysts.*[a]

Buffer	pH	CTA	Oocysts	Mean A_{520} ± SEM	n
Phosphate	7.0	Yes	No	0.256 ± 0.027	8
Phosphate	7.0	Yes	Yes	0.209 ± 0.022	8
Phosphate	7.0	No	No	0.257 ± 0.058	2
Tris	9.0	Yes	No	0.502 ± 0.052	5
Tris	9.0	Yes	Yes	0.498 ± 0.021	10
Tris	9.0	No	No	0.385 ± 0.115	2
Acetate	5.0	Yes	No	0.138 ± 0.017	8
Acetate	5.0	Yes	Yes	0.131 ± 0.018	8
Acetate	5.0	No	No	0.102 ± 0.047	2

[a] Values for oocyst and no oocyst groups at the same pH are not significantly different ($P < 0.05$) from each other or from buffer-only controls.





Figs. 13, 14. Effects of protease inhibitors on excystation of bleach-treated oocysts. Excystation conditions as in Fig. 6. 13. Pepstatin, experiment similar to Fig. 9. 14. Leupeptin, experiment similar to Fig. 9.

trypsin provided that the sporocysts were treated with cysteine/CO_2. Thus, it is possible that other suture-bearing coccidia also use an alternate excystation mechanism.

Existence of parasite-derived enzymatic pathways for excystation has been proposed for *Cryptosporidium* (5) and other coccidians (8, 9). The evidence suggests that enzymes are involved in excystation of *Cryptosporidium* since the process is temperature dependent and responsive to changes in pH. Excystation of bleach-treated oocysts requires only a rise in temperature and, therefore, must be effected by mechanisms within the oocyst. Reduker et al. (12) showed that morphological changes that occur in the oocyst wall during excystation appear first on the internal aspects of the excystation suture, further supporting the idea that excystation is effected from inside the oocyst.

The current experiments raise important questions about the mechanism of excystation in *Cryptosporidium* and perhaps other suture-bearing coccidia. The data suggest that temperature, oxidation/reduction reactions, and bile salts may serve as triggers for excystation in vivo, but that these triggers do not just pave the way for digestion of the cyst wall by host proteolytic enzymes. Rather, they may activate an enzymatic system inside the oocyst which, in turn, effects excystation.

LITERATURE CITED

1. Chobotar, B. & Scholtyseck, E. 1982. Ultrastructure, *in* Long, P. L., ed., *The Biology of the Coccidia.* University Park Press, Baltimore, pp. 101–165.

2. Current, W. L. & Reese, N. C. 1986. A comparison of endogenous development of three isolates of *Cryptosporidium* in suckling mice. *J. Protozool.*, 33: 98–108.

3. Current, W. L., Reese, N. C., Ernst, J. V., Bailey, W. S., Heyman, M. B. & Weinstein, W. M. 1983. Human cryptosporidiosis in immunocompetent and immunodeficient persons. *N. Engl. J. Med.*, 308: 1252–1257.

4. Fayer, R. & Leek, R. G. 1973. Excystation of *Sarcocystis fusiformis* sporocysts from dogs. *Proc. Helminthol. Soc. Wash.*, 40: 294–296.

5. ——— 1984. The effects of reducing conditions, medium, pH, temperature, and time on *in vitro* excystation of *Cryptosporidium*. *J. Protozool.*, 31: 567–569.

6. Heine, J., Moon, H. W. & Woodmansee, D. B. 1984. Persistent *Cryptosporidium* infection in congenitally athymic (nude) mice. *Infect. Immun.*, 43: 856–859.

7. Heine, J., Pohlenz, J. F. L., Moon, H. W. & Woode, G. N. 1984. Enteric lesions and diarrhea in gnotobiotic calves monoinfected with *Cryptosporidium* species. *J. Infect. Dis.*, 150: 768–775.

8. Hibbert, L. E. & Hammond, D. M. 1968. Effects of temperature on *in vitro* excystation of various *Eimeria* species. *Exp. Parasitol.*, 23: 161–170.

9. Jackson, A. R. B. 1962. Excystation of *Eimeria arlongi* (Marotel, 1905): stimuli from the host sheep. *Nature*, 194: 847–849.

10. Jolley, W. R., Burton, S. D., Nyberg, P. A. & Jensen, J. B. 1976. Formation of sulfhydryl groups in the walls of *Eimeria stiedai* and *E. tenella* oocysts subjected to *in vitro* excystation. *J. Parasitol.*, 62: 199–202

11. Reduker, D. W. & Speer, C. A. 1985. Factors influencing excystation in *Cryptosporidium* oocysts from cattle. *J. Parasitol.*, 71: 112–115.

12. Reduker, D. W., Speer, C. A. & Blixt, J. A. 1985. Ultrastructural changes in the oocyst wall during excystation of *Cryptosporidium parvum* (Apicomplexa; Eucoccidiorida). *Can. J. Zool.*, 63: 1892–1896.

13. Ryley, J. F. 1973. Cytochemistry, physiology, and biochemistry, *in* Hammond, D. M. & Long, P. L., eds., *The Coccidia:* Eimeria, Isospora, Toxoplasma, *and Related Genera.* University Park Press, Baltimore, pp. 145–181.

14. Sloss, M. W. & Kemp, R. L. 1978. *Veterinary Clinical Parasitology.* Iowa State University Press, Ames, Iowa, p. 8.

15. Sundermann, C. A., Lindsay, D. S. & Blagburn, B. L. 1987. *In vitro* excystation of *Cryptosporidium baileyi* from chickens. *J. Protozool.*, 34: 28–30.

16. Tyzzer, E. E. 1912. *Cryptosporidium parvum* (sp. nov.) a coccidium found in the small intestine of the common mouse. *Arch. Protistenkd.*, 26: 394–418.

17. Tzipori, S. 1983. Cryptosporidiosis in animals and humans. *Microbiol. Rev.*, 47: 84–96.

18. Upton, S. J. & Current, W. L. 1985. The species of *Cryptosporidium* (Apicomplexa: Cryptosporidiidae) infecting mammals. *J. Parasitol.*, 71: 625–629.

19. Wang, C. C. 1982. Biochemistry and physiology of Coccidia, *in* Long, P. L., ed., *The Biology of the Coccidia.* University Park Press, Baltimore, pp. 167–228.

20. Woodmansee, D. B. & Pohlenz, J. F. L. 1984. Development of *Cryptosporidium* sp. in a human rectal tumor cell line. *Proc. 4th Int. Symp. Neonatal Diarrhea.* Veterinary Infectious Disease Organization, Saskatoon, Saskatchewan, pp. 306–317.

Received 7 1 87; accepted 15 VI 87